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PHARMACOTHERAPY OF EBOLA HEMORRHAGIC FEVER: A BRIEF REVIEW OF CURRENT STATUS AND FUTURE PERSPECTIVES

Abstract: The 2014 outbreak clearly showed that Ebola viruses (EBOV) remain a substantial threat for public health. The mainstay of management of patients with Ebola disease is isolation of patients and use of strict barrier nursing procedures; the present treatment strategies are mainly symptomatic and supportive (fluid resuscitation, antipyretics, antidiarrheal drugs). Currently, there is no approved therapy for Ebola hemorrhagic fever (EHF), however several advanced treatment options were tested in animal models (on non-human primates or rodents). They include use of both symptomatic (e.g. use of tissue factor inhibitors — rhNAPc2, rhAPC — to abolish coagulopathy) and specific antiviral approaches: e.g. monoclonal anti EBOV antibodies (ZMapp, MB-003), phosphorodiamidate morpholino oligomers (PMOs), liposomes containing siRNA (LNP-siRNA:TKM-Ebola) and small molecule inhibitors (e.g. BCX4430, favipiravir). The scope of this article is to briefly review the most promising therapeutics for EHF, based on the data coming from rare clinical reports, studies on animals and results from in vitro models.

Key words: Ebola, hemorrhagic fever, treatment methods.

PHARMACOBIOLOGY OF EBOLA VIRUS INFECTION

Ebola virus (EBOV), the culprit of current deadly epidemic in West Africa, has been one of the most feared pathogens, causing severe hemorrhagic fever (EHF) with high mortality [1]. The genus *Ebolavirus* (family: *Filoviridae*, order: *Mononegavirales*) includes five species: *Zaire ebolavirus* (ZEBOV), *Sudan ebolavirus* (SUDV), *Reston ebolavirus* (RESTV), *Tai Forest ebolavirus* (TAFV) and *Bundibugyo ebolavirus* (BDBV), each pathogenic for humans except RESTV, which has been shown to be pathogenic only for non-human primates [2]. All EBOV species have been sequenced and their evolutionary characteristics have been reported [3]. The EBOV is a lipid enveloped virus of filamentous shape with a mean unit length of 1200 nm [3]. The single 19-kb negative sense RNA encodes seven proteins: nucleoprotein (NP), glycoprotein (GP, produced also in its soluble truncated form — sGP, through RNA editing), RNA dependent RNA polymerase (L) and four matrix proteins (VP24,

VP30, VP35 and VP40). The inner matrix proteins — VP30 and VP35 and the polymerase (L) form the complex involved in transcription and replication, while the VP40 and VP24 are linked to the ribonucleoprotein complex at the inner surface of lipid envelope, which is originated from the host cell [2]. The viral envelope contains only the highly glycosylated glycoproteins, assembled after cleavage of precursor polyprotein by cellular furin-like enzyme, as a trimeric spikes consisting of two fragments: extracellular protein (GP1) and membrane-anchored protein (GP2). While the GP1 is believed to play sole crucial role in virus attachment to the various host cells, several potential candidates for GP1 receptor/co-receptors on cells have been characterized: transferrin, folate receptor, members of the Tyro3 family of receptor tyrosine kinases (Axl, Dtk, Mer), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and T-cell immunoglobulin and mucin domain (TIM-1) proteins [4, 5]. Following binding the virus is taken up into the cells via micropinocytosis. The cholesterol transport protein Niemann-Pick C1 (NPC1) seems to act as an intracellular receptor for EBOV after cleavage of viral GP by low PH-dependent cathepsins B and L. Such diversified entry mechanisms surely account for broad cell tropism of EBOV [6]. It is generally accepted that monocytes/macrophages and dendritic cells are primary targets for EBOV, however virus quickly disseminates to endothelial cells and subsequently can be found in various organs (lymph nodes, spleen, liver). Such spreading coincides with dysregulation of the immune/inflammatory mechanisms (fever, cytokine storm, complement activation, overproduction of nitric oxide etc.), severe endothelial injury (vascular leakage, bleeding), liver failure (hepatocellular necrosis, coagulopathy, DIC) and adrenal gland dysfunction (impairment of corticosteroid production). Clinically, EBOV infection is characterized by abrupt onset of flu-like symptoms after incubation period of 2–21 days. These are accompanied by anorexia and gradually increasing nausea, emesis and diarrhea. In few days 25–52% of patients develop maculo-papular rash and petechiae. Swelling of lymph nodes as well as enlargement of kidneys, testes/ovaries or brain edema may occur. In later stages, patients demonstrate hypotension and shock, mucosal hemorrhages (typically from GI tract or conjunctival) and multi-organ failure (particularly renal and liver). Case fatality rates associated with EBOV infection range between 50–90% and mainly depend on virus species, with ZEBOV being the most deadly. Long period of convalescence is often associated with complications like hepatitis, uveitis, myelitis and psychotic reactions [1, 2].

The great role in pathobiology of EHF plays deregulated, exaggerated response of host organism. EBOV infection quickly triggers massive expression of inflammatory mediators including nitric oxide and reactive oxygen species (which are partially responsible for microvascular complications) and a whole bunch of pro- and anti-inflammatory proteins: interferons, interleukins (e.g. IL-2, 6, 8, 10), tumor necrosis factor alpha (TNF α), chemokines (e.g. macrophage chemoattractant protein-1) leading to immunological paralysis that contributes to the progression

of disease [1, 2]. Noteworthy, Ebola virus developed multiple mechanisms of disabling of effective, immune anti-viral response (e.g. VP24 and VP34-dependent inhibition of interferon expression and action in host cells, epitope shielding due to GP glycosylation, down-regulation of MHC-class II and co-stimulatory molecules on dendritic cells) [7]. An important difference between EBOV and other *Mononegavirales* is the production of large quantities of soluble GP, however its exact pathological role in EHF remains unresolved.

MANAGEMENT STRATEGIES

The mainstay of current management is isolation of patients and use of strict barrier nursing procedures. Present treatment strategies are mainly symptomatic and supportive. In general, the typical antiviral drugs like interferons (except slight positive effect of beta-interferone [8]) or ribavirin are not effective [9]. Although a number of effective chemotherapeutic/chemopreventive agents have been identified in various animal models – on Non-Human Primates (NHPs) and Small Animal Models (SAM) — as well as several interesting compounds have been found to interfere with EBOV life cycle in cultured cells *in vitro*, by far there is no approved specific therapy for EHF.

SYMPTOMATIC AND SUPPORTIVE CARE

The critical analysis of various supportive regimens documented in published reports after 34 recognized EHF outbreaks since 1967 was provided by Clark *et al.* [10]. As the early EHF signs are difficult to distinguish from other febrile infections, patient care typically followed the use of antipyretics (paracetamol, aspirin, dipyrrone), antibiotics (tetracycline, chloramphenicol, penicillin, ampicillin, ciprofloxacin) and antimalarial drugs (chloroquine, amodiaquine, pyrimethamine, atovaquon-proguanil, artemisinin combination therapy). Antibiotics were given also later in the course of disease to prevent or treat secondary bacterial infections. Other symptomatic treatment, depending on clinical needs, included oral/intravenous fluid therapy, antidiarrheal drugs, antiemetics, analgesics, sedatives and antipsychotic drugs to reduce anxiety and agitation. In severe cases treatment included transfusion of blood (from regular donors or convalescent patients) and blood products (whole blood, packed red blood cells, fresh frozen plasma, platelets) and various blood-derived regulators of coagulation (clotting factors, fibrinogen, prothrombin, proconvertin, Stuart-factor, antihemophilic globulin B). Anti-coagulants (heparin) and rheological agents (pentoxifylline) were administered to some patients to prevent/treat disseminated intravascular coagulation. There are no clinical trials providing evidence-based guidance regarding basic supportive

care. The data coming from the studies on rhesus macaques suggest that fluid replacement may limit renal injury, but has no impact on overall mortality [11, 12]. It is generally accepted, that as pathological process of EHF resembles that of severe sepsis, therefore aggressive supportive care protocols, with early fluid resuscitation may represent reasonable strategy of treatment. However, it should be noted that aggressive fluid therapy, given endothelial injury in EHF patients, may pose the risk of pulmonary edema [10].

Severe coagulation disorder, developing due to massive exposure of tissue factor (TF) on injured endothelium is one of the most prominent features of advanced EHF, therefore the possibility of inhibiting this pathway has been considered as an important therapeutic approach. In this regard, agents that may reduce mortality without influencing directly virus replication include recombinant nematode anticoagulant protein c2 (rNAPc2) and recombinant human activated protein C (rhAPC), inhibitors of factor VIIa-TF complex [13, 14]. Geisbert *et al.* demonstrated that in rhesus macaques infected with EBOV, early post exposure treatment with rNAPc2 (daily s.c. injection of 30 µg/kg) provided partial protection, as it resulted in 33% survival rate and 3,4-day increase in time-to-death in treated animals in a typically 100% lethal model [15]. Importantly, rNAPc2 is currently in phase II clinical trials in orthopedic surgery and coronary revascularization and shows good safety record.

The experiments with EBOV in NHPs demonstrated significant decrease in circulatory levels of protein C [16]. The recombinant human activated protein C (drotrecogin alpha, rhAPC) given 30–60 min after EBOV infection of rhesus macaques and continuing for next 7 days (in daily s.c. injections of 30 µg/kg) provided 20% survival and almost 4-day increase of in time-to-death of treated animals. The current status of rhAPC is unclear — it was formerly approved for the treatment of patients with advanced septic shock, however taking into granted the risk of adverse effects (bleeding) the Cochrane Review in 2011 not recommended clinical use the hrAPC in this indication [14].

Thus, both TF inhibitors represent promising advanced symptomatic therapies (not directly targeting the virus) and should be probably considered for use in EHF treatment in conjunction with specific anti-EBOV compounds.

SPECIFIC ANTI-EBOV TREATMENT

An attractive treatment option that has been applied to EHF is the use of passive immunization, however the first attempts were not successful: despite some positive effects in SAM, antibody treatment with equine immunoglobulin against EBOV or with recombinant human monoclonal antibody did not protect NHPs [16]. Human convalescent blood or serum has been used in NHPs, but the results were controversial [17]. During 1995 outbreak in Kikwit (DRC), human convalescent

plasma was given to 8 patients with proven Ebola disease and only 1 patients died, however subsequent studies could not confirm clear benefit conferred by convalescent plasma. More recent studies have demonstrated protection in SAM and NHPs with use of polyclonal and monoclonal antibodies [18]. The antibodies targeting EBOV GP mucin-like domain have been shown to protect 100% mice against lethal EBOV challenge [19].

The most effective approach to post-exposure immunotherapy consists on use of cocktails of monoclonal antibodies against epitopes of EBOV. Two cocktails demonstrated significant protection in NHPs against EBOV infection: MB-003 and ZMapp.

MB-003 comprise of three monoclonal antibodies, which were raised in mice immunized with virus of Venezuelan equine encephalitis containing GP (VEEV-GP) [20]. The antibodies 13C6 and 6D8 neutralized EBOV *via* complement pathway, while 13F6 was non-neutralizing. For further studies these antibodies were produced either as a human-mouse chimera (c) or humanized (h) form in the tobacco plant (*Nicotiana benthamiana*). MB-003 was tried in rhesus macaques (50 mg/kg *i.v.*) beginning at 24 or 48 hours after challenge, with three identical doses given every 2–3 days. Such treatment provided 67% protection [20].

Another product — ZMapp — containing 3 chimeric monoclonal antibodies against EBOV GP epitopes (c13C6, c2G4 and c4G7), each produced in large quantities in *Nicotiana benthamiana* recently has been shown to confer survival benefit in NHPs when administered even 4–5 days after infection [21]. This formulation was administered to three patients in 2014 and the conditions of two reportedly improved soon after they received it, while the third patient died [22]. Clearly more clinical experience is needed to clarify the question of efficacy of such treatment in EHF patients. Nevertheless, the U.S. government (NIH's National Institute of Allergy and Infectious Diseases, The Department of Defense's: Defense Threat Reduction Agency and the HHS'Biomedical Advanced Research and Development Authority) has provided support for the development of this experimental treatment.

Next to post exposure immunotherapy several other EBOV-specific therapeutics are under investigation in NHPs and SAM. Phosphorodiamidate morpholino oligomers (PMOs) are cell permeable nucleotide analogs, which bind specific sequences of EBOV mRNA and by steric hindrance prevent the translation of the viral mRNA [23]. The morpholino oligomers have been previously demonstrated highly stable, safe and effective therapeutics against coronaviruses and flaviviruses [24]. PMOs targeting EBOV VP24 and EBOV VP35 mRNAs have shown efficacy in mice and guinea pigs models of EBOV infection [25]. Moreover, AVI-6002 (a combination of PMOs against EBOV VP24 and EBOV VP35 mRNAs), delivered 30–60 min post-exposure (and daily *s.c./i.p.* or in *i.v.* injection of 40 mg/kg) protected >60% of rhesus macaques against lethal EBOV infection [23]. Clearly, PMOs due to its efficacy and good safety record may represent a viable therapeutic strategy in EHF. Currently, AVI-6002 is in phase I clinical trials.

Another strategy to inhibit EBOV replication is use of small interfering RNAs (RNAi), which interfere with translation by either sterically blocking specific mRNA or by triggering RNase H-mediated cleavage of the DNA/RNA duplex with subsequent inhibition of gene expression. The siRNA targeting the EBOV RNA polymerase L protein, encapsulated in bilayered liposomes, forming stable nucleic acid particles (SNALPs) has been developed by Tekmira Inc. (LNP-siRNA:TKM-Ebola) [26]. The SNALPs targeting EBOV polymerase L, VP24 and VP35 were tried on rhesus macaques in four or seven doses following lethal challenge with EBOV. Two of three monkeys given four doses survived, while all four monkeys given seven doses survived otherwise lethal infection. Importantly, there were no evidence for serious side effects of that treatment, except mild elevations of liver transaminases. Currently, LNP-siRNA:TKM-Ebola is in phase I clinical trials.

Only recently, a broad spectrum nucleoside analog BCX4430 (Immucillin-A) developed by BioCryst Pharmaceuticals in association with U.S. Army showed significant protection against wide variety of viruses, including EBOV in post-exposure models in NHPs [27]. This compound inhibits viral polymerase function and acts as a non-obligate RNA chain terminator. In rhesus macaques challenged with EBOV, at a dose of 15 mg/kg, starting either 1, 24, and 48 hours after infection and continuing twice daily for next 14 days BCX4430 protected 83% animals in the 1-hour group, and 100% in the 24- and 48-hour groups [27]. Regarding high efficacy (but on the other hand the high number of doses required for protection) the issue of drug-related adverse effects should be obligatory addressed before clinical trials.

Several treatment options, not tested in NHPs so far, have shown efficacy in small animal models of EBOV infection. Endogenous plasma mannose-binding lectin (MBL), produced in the liver recognizes carbohydrate moieties (mannose, hexose) of external surface of microbes (pathogen associated molecular patterns, PAMPs), which are not present on host cells. Importantly, EBOV contains large amounts of mannose in GP, thus upon binding with the MBL it is inactivated through the lectin-dependent of complement cascade [28]. Human recombinant MBL (350 µg *i.p.* twice daily for 10 days) has been shown to protect 40% of mice exposed to EBOV [29].

Several small molecule inhibitors have also shown some efficacy in SAM of EBOV infection. A pyrazinecarboxamide derivative T-705 (favipiravir) developed by Toyama Chemical of Japan was reported to inhibit *in vitro* EBOV RNA-dependent RNA polymerase and clear *in vivo* virus infection within four days of treatment initiated at day 6 post infection [30, 31]. Favipiravir was given in October 2014 to the French nurse, who contracted Ebola disease in Liberia and fully recovered. Notably, the compound JK-05 developed by Chinese company Sihuan Pharmaceutical with support of the Chinese Army, similar to favipiravir was available for Chinese health workers involved in 2014 West African Ebola outbreak.

A variety of compounds with unclear mechanisms of action, like metal ion-based therapeutic hexamminecobalt (III) chloride (Cohex), heterocyclic aromatic structures targeting late event in EBOV replication cycle — FGI-103, FGI-104, FGI-106, and reactive oxygen species scavenger — NSC 62914 have shown inhibitory actions on EBOV replication *in vitro* and protective effects in pre- and/or post-exposure treatment in small animal models of EBOV infection [18, 32]. Interestingly, several adenosine analogues have been shown to inhibit the replication of Ebola virus *in vitro*, most probably by blocking the S-adenosyl-L-homocysteine hydrolase and thereby indirectly limiting methylation of the 5' cap of viral mRNA. Noteworthy, mice treated thrice daily for 9 days (2.2–20 mg/kg) with carbocyclic 3-deazaadenosine, were protected against lethal EBOV challenge, moreover, it appeared that even single administration of the same substance, or another analogue, 3-deazaneplanocin A, provides equal or better protection, without causing acute toxicity [33]. Despite very promising results of studies on SAM, the effectiveness of all these compounds in NHPs remain to be tested.

A whole bunch of compounds showed *in vitro* activity against EBOV.

Brincidofovir (CMX001) is a prodrug — lipid conjugate of cidofovir, developed by Chimerix (Durham, NC) for the treatment of herpes simplex, cytomegalovirus, adenovirus and smallpox infections (currently in phase III clinical trials). It is generally active against dsDNA viruses, however preliminary *in vitro* tests have shown its efficacy against EBOV, which is somehow surprising, as EBOV is not a DNA virus [34]. In October 2014 Chimerix received FDA approval for emergency investigational new drug application for the treatment of Ebola disease and same month was administered to two patients: first, critically ill patient, was given brincidofovir 6 days after onset of infection; he died four days later. The second patient, released from Nebraska Medical Center was pronounced Ebola-free. Recently, brincidofovir as potential anti-EBOV drug entered phase II clinical trials.

LJ001, an aryl methyldiene rhodamine derivative was identified *in vitro* as efficient virus-cell entry inhibitor for variety of enveloped viruses (EBOV, HCV, HIV-1) [32]. Although, initial testing in mice did not show significant activity, possible further improvement of pharmacokinetic properties makes this compound promising future therapeutic.

Several compounds targeting molecules/pathways important for EBOV entry into cells have shown the efficacy *in vitro*: the inhibitors of Niemann Pick C1 (NPC1) protein (e.g. U18666A), inhibitors of cathepsin L or B, HSP90 inhibitors [18, 32, 35]. There were also identified several anti-EBOV compounds with unclear mechanisms of action: Δ -peptide immunoadhesins (possibly interfering with GP processing in cells), C-peptides (possibly preventing fusion of the virus to the host cell membrane), compound 7 (benzodiazepine derivative, possibly inhibiting early stages of virus entry to the cells) [18, 32].

Only recently, microRNA inhibitors (hsa-miR-1246, hsa-miR-320a and hsa-miR-196b-5p) were found to ameliorate GP-dependent cell damage *in vitro* [36].

This observation points to the novel mechanisms of EBOV cellular toxicity and possibly, opens new way for design of EHF therapies.

Interestingly, several known drugs approved for treatment of various diseases clearly showed strong *in vitro* anti-EBOV activities. Recently, estrogen receptor modulators (clomiphen, toremiphen) and several anti-dysrhythmic drugs (amiodarone, dronedarone, verapamil) have been reported to inhibit EBOV cell entry, possibly by interfering with NPC1 activity. Also, antimalarial drug — chloroquine has been shown to disrupt EBOV entry and replication *in vitro*, as well as increase survival rate in mice challenged with lethal EBOV infection [37–39]. Very recently, extensive search revealed over 50 drugs able to interfere with EBOV replication cycle in vitro [40]. Such observations triggered debate about the allowing fast track use of existing medicines, with known safety profiles as “repurposed” drugs for Ebola treatment [41].

Over the last few years we have witnessed the great advances in the development of EBOV therapeutics. Although the most notably progress was done on the field of vaccines, also several promising post-exposure candidate drugs were developed.

Table 1

Potential specific anti-EBOV therapeutics showing efficacy in non-human primates (NHPs), small animal models (SAM) and *in vitro*. Detailed description — see text.

Efficient in NHPs	ZMapp (cocktail of monoclonal antibodies) MB-003 (cocktail of monoclonal antibodies) Phosphoroamidate Morpholino Oligomers (PMOs) LNP-siRNA:TKM-Ebola BCX4430
Efficient in SAM	rhMBL pyrazinecarboxamide derivative T-705 (favipiravir) JK-05 hexammincobalt (III) chloride (Cohex) FGI-103, FGI-104, FGI-106 NSC 62914 3-deazaneplanocin A
Efficient <i>in vitro</i>	Brincidofovir LJ001 NPC1 inhibitors (U18666A) selected repurposed drugs (chloroquine, amiodarone, dronedarone, clomiphen, toremiphen) HSP90 inhibitors Δ-peptide immunoadhesins C-peptides benzodiazepine derivative (compound 7) specific microRNA inhibitors

There is a cause of optimism, that despite the ethical issues regarding use of investigational drugs in humans and problems with design and organization of clinical trials with patients with hemorrhagic fever, some of these drugs soon will become more widely available for emergency use and commercially available within next 5–10 years.

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